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Interactions between cancer cells and their associated stroma can influence the initiation, progression, and overall prognosis of breast cancer. The most predominant stromal cell type implicated in breast cancer progression is the mammary fibroblast. These cells, which normally provide structural integrity and extracellular matrix remodeling required for proper mammary gland development, transition to an "activated" state (now referred to as cancer associated fibroblasts, CAFs) to promote breast tumor growth and survival. However, the cell-intrinsic mechanisms regulating mammary fibroblast activation remain poorly understood. We sought to determine if autophagy regulates the tumor suppressive or tumor promoting behavior of mammary fibroblasts. To do so, we utilized an immunocompetent, cleared mouse mammary gland transplantation model to engraft autophagy deficient or autophagy competent fibroblasts into their native microenvironment. Fat pads "primed" with autophagy deficient fibroblasts show reduced recruitment of CD3+ cells. Moreover, these fat pads fail to support PyMT tumor growth as compared to fat pads primed with autophagy competent fibroblasts. The tumor suppressive behavior of autophagy deficient fibroblasts is contingent upon an intact adaptive immune response, as this phenotype is masked in immunodeficient mice. We are currently investigating how fibroblast specific autophagy competency modulates the tumor microenvironment in a manner permissible for tumor growth. Our data suggests stromal autophagy contributes to the development and progression of mammary tumors, and advocates for the use of autophagy inhibitors as an adjuvant therapy in treating both early and late stage breast cancers.

**15. SUBJECT TERMS**
fibroblast, cancer associated fibroblast (CAF), autophagy, Atg12, FSP-Cre, desmoplasia, breast cancer, collagen

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# INTRODUCTION

Breast cancer is a heterogeneous, multi-factorial disease of aberrant breast development whose etiology relies upon microenvironmental changes within the tissue. Such changes involve the appearance of $\alpha$-smooth muscle actin positive ($\alpha$SMA+) fibroblasts (referred to as "cancer associated fibroblasts", or CAFs), recruitment of various immune cells (macrophages, T cells, B cells, T regulatory cells), and enhanced type I collagen deposition [1]. Within the last decade, a plethora of evidence demonstrates the importance of this inflammatory and desmoplastic stromal response to the initiation and progression of breast cancer [2]. In particular, CAFs are the major cell type responsible for tumor desmoplasia, as these cells secrete abundant levels of type I collagen which alters the stiffness and compliance of the tumor associated extra-cellular matrix (ECM) [3]. ECM stiffening, as a result of CAF-mediated collagen deposition, enhances integrin and PI3K signaling, both of which support mammary tumor initiation and progression [4]. While the functional contribution of CAFs to breast cancer progression is well documented, the molecular mechanisms and cellular pathways that govern CAF behavior remain poorly understood. Importantly, CAFs are exposed to the same hypoxic and nutrient deprived microenvironments as tumor cells [5]; thus, they most likely upregulate stress response pathways in order to survive under these conditions. A commonly upregulated stress response pathway is autophagy, an evolutionarily conserved cellular stress response pathway that serves to remove damaged proteins and organelles and prevent cellular toxicity. The process involves the formation of a double membrane organelle, the autophagosome, which sequesters cytoplasmic contents and fuses with the lysosome for degradation and metabolite recycling [6, 7]. Several cancer-relevant stresses induce autophagy, including nutrient deprivation, hypoxia, the unfolded protein response, mechanical stress and chemotherapy [6, 8]. Because autophagy most often functions as a cellular survival mechanism, autophagy inhibitors in conjunction with chemotherapy could hold tremendous promise in the eradication of breast cancer [9-12]. Currently, the autophagy inhibitor hydroxychloroquine (HCQ) is in phase I and II clinical trials as an adjuvant therapy for the treatment of metastatic breast cancer [13, 14]. **However, it remains largely unknown how autophagy inhibition alters the behavior of stromal cell types, and how this ultimately impacts the growth and survival of breast tumor cells.** By utilizing mice harboring floxed alleles of essential autophagy genes, I will determine how tissue specific deletion of autophagy in stromal fibroblasts impacts mammary tumor development.

# KEYWORDS

fibroblast, cancer associated fibroblast (CAF), autophagy, Atg12, FSP-Cre, desmoplasia, breast cancer, collagen
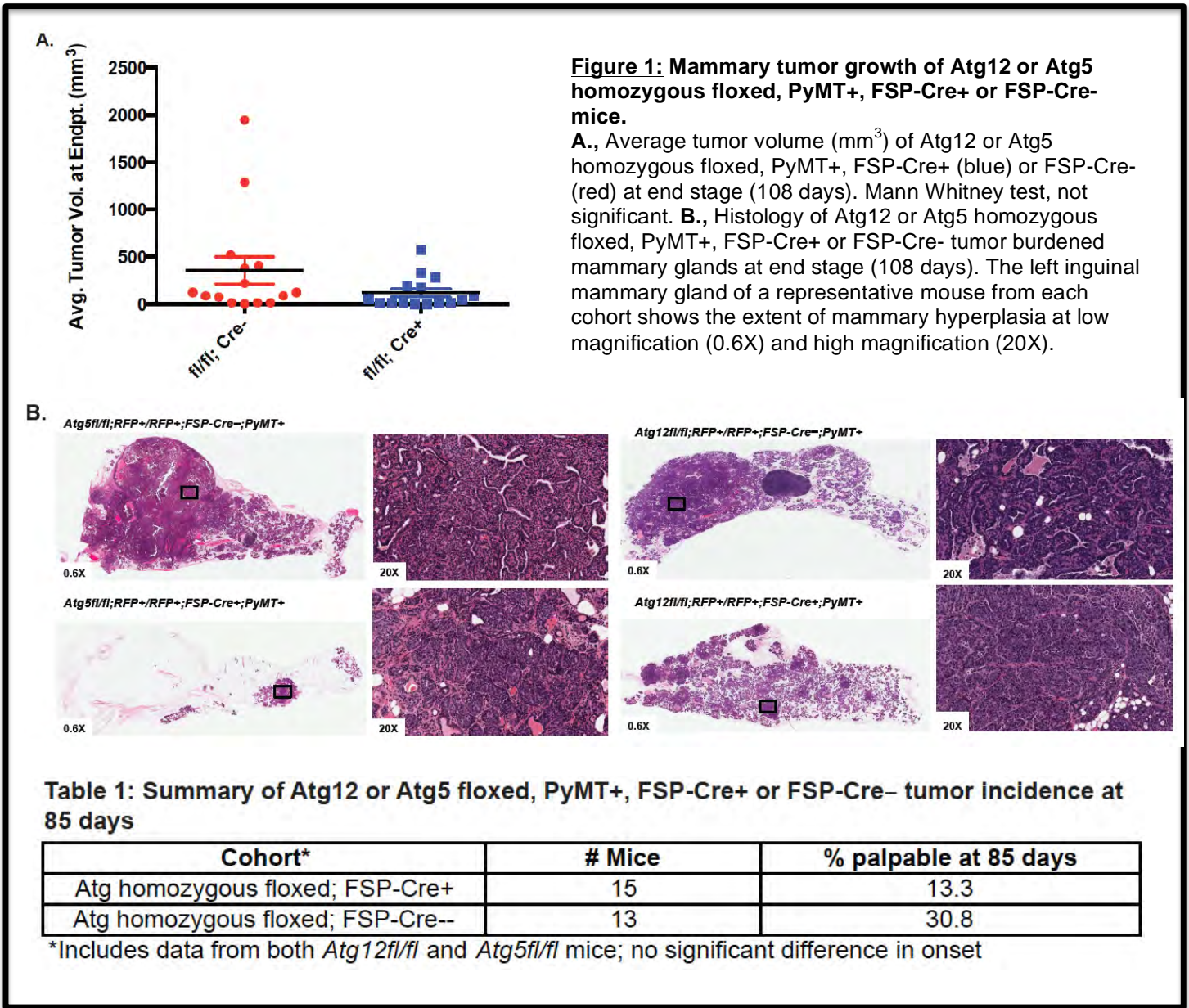
# ACCOMPLISHMENTS

In accordance with the approved Statement of Work for this proposal, 6 subtasks were assigned to Year 2. Given that the transgenic mice generated in Task 1b did not show significant differences in tumor growth and latency (see Figure 1), we have amended our experimental design to a pure C57B/6 transplantation model of mammary tumorigenesis.

***Task 1: To determine the effects of stromal fibroblast specific atg deletion on mammary tumor progression.***

***Subtask 1c: Determine the latency period for the onset of primary tumor formation and metastasis for the transgenic mice generated in Subtask 1b (months 6-30).***

In Year 1, we generated quadruple transgenic mice harboring homozygous floxed alleles of Atg12 or Atg5 with a lox-stop-lox RFP reporter, PyMT antigen, and FSP driving Cre-recombinase (*Atg12fl/fl;lsl-RFP+;PyMT+;FSP-Cre+* and *Atg5fl/fl;lsl-RFP+;PyMT+;FSP-Cre+*). These mice were generated on a mixed background. Mammary tumor development for each genotype was compared to the *FSP-Cre–* cohort for each Atg (*Atg12fl/fl;lsl-RFP+;PyMT+;FSP-Cre–* and *Atg5fl/fl;lsl-RFP+;PyMT+;FSP-Cre–*). Both the *Atg12fl/fl;lsl-RFP+;PyMT+;FSP-Cre+* and the *Atg5fl/fl;lsl-RFP+;PyMT+;FSP-Cre+* cohorts showed similar tumor growth kinetics and histology. Given these data, and the fact that we are still generating sufficient numbers in the Atg5 cohort for statistical significance, we have combined Atg12 and Atg5 tumor growth data to date, classifying the cohorts as either *FSP-Cre+* or *FSP-Cre–*. Despite a trending reduction in endpoint tumor volume, Atg12 and Atg5 homozygous floxed, PyMT+, FSP-Cre+ mice do not show a significant difference in mammary tumor growth compared to the autophagy competent, *FSP-Cre–* control cohort (Figure 1A). Both cohorts of mice demonstrate extensive mammary hyperplasia in glands that are not yet tumor burdened (108 day timepoint)

(Figure 1B). Both cohorts of mice exhibit similar onset for palpable tumor detection (Table 1). These data suggest that using this model system, stromal autophagy deletion (mediated by FSP driven Cre recombinase) does not significantly alter mammary tumor onset and latency. Moreover, fluorescence activated cell sorting (FACS) data suggests that *FSP-Cre* activity is restricted to the stromal compartment of female mouse
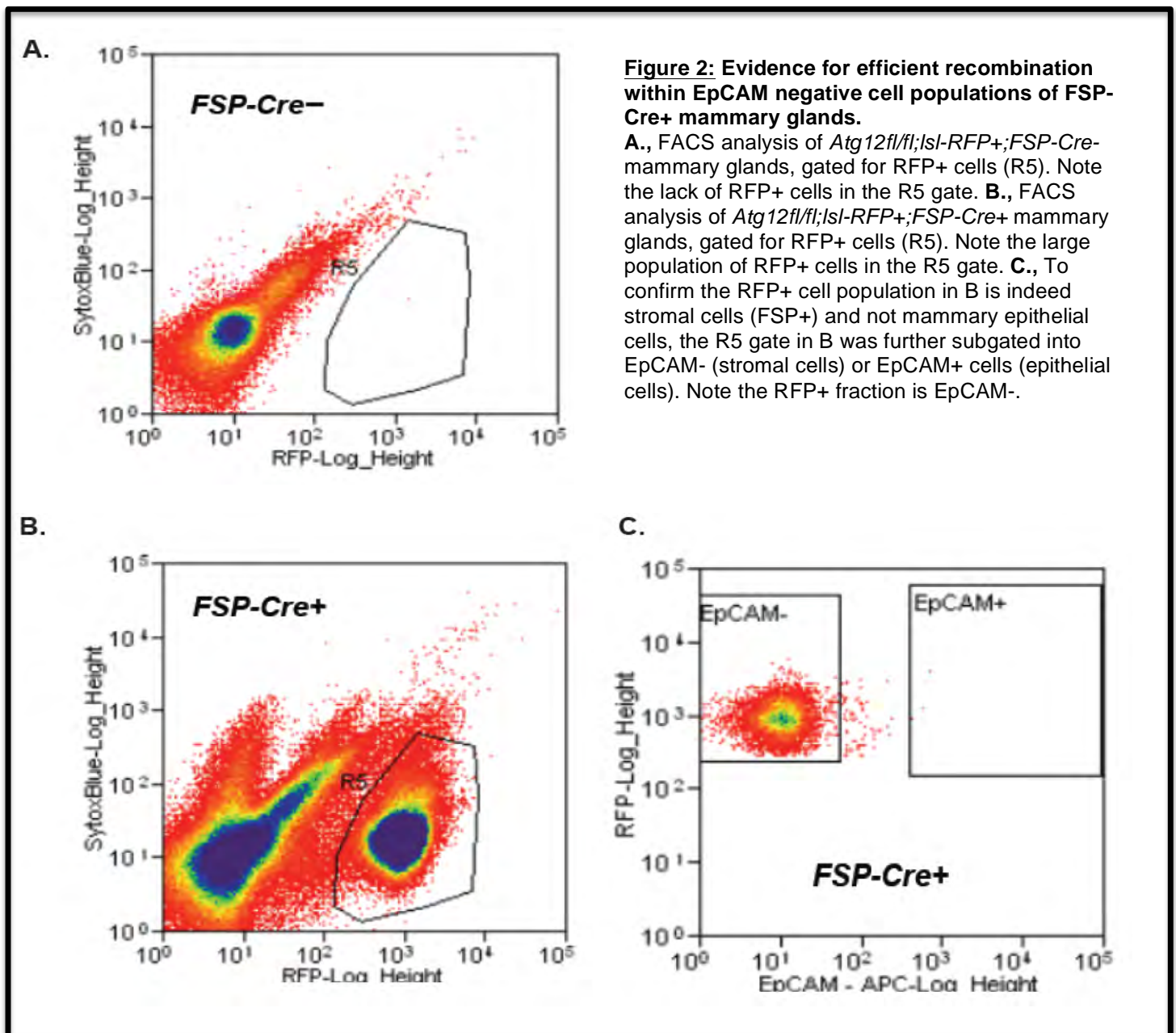


**A.**

**Figure 1: Mammary tumor growth of Atg12 or Atg5 homozygous floxed, PyMT+, FSP-Cre+ or FSP-Cre-mice.**
**A.,** Average tumor volume (mm$^3$) of Atg12 or Atg5 homozygous floxed, PyMT+, FSP-Cre+ (blue) or FSP-Cre-(red) at end stage (108 days). Mann Whitney test, not significant. **B.,** Histology of Atg12 or Atg5 homozygous floxed, PyMT+, FSP-Cre+ or FSP-Cre- tumor burdened mammary glands at end stage (108 days). The left inguinal mammary gland of a representative mouse from each cohort shows the extent of mammary hyperplasia at low magnification (0.6X) and high magnification (20X).

**B.**

Atg5fl/fl;RFP+/RFP+;FSP-Cre−;PyMT+

Atg12fl/fl;RFP+/RFP+;FSP-Cre−;PyMT+

Atg5fl/fl;RFP+/RFP+;FSP-Cre+;PyMT+

Atg12fl/fl;RFP+/RFP+;FSP-Cre+;PyMT+

**Table 1: Summary of Atg12 or Atg5 floxed, PyMT+, FSP-Cre+ or FSP-Cre– tumor incidence at 85 days**

| Cohort* | # Mice | % palpable at 85 days |
|---|---|---|
| Atg homozygous floxed; FSP-Cre+ | 15 | 13.3 |
| Atg homozygous floxed; FSP-Cre-- | 13 | 30.8 |

*Includes data from both *Atg12fl/fl* and *Atg5fl/fl* mice; no significant difference in onset

mammary glands, given that RFP+ cells are contained in the EpCAM– cell fraction (Figure 2), which enriches for stromal cells and omits mammary epithelial cells (MECs).

We are currently continuing these breedings and hope to generate n=20 mice per Atg cohort, upon which we will perform a final statistical analysis.

***Subtask 1e: Perform co-mixing experiments with CAFs isolated in Subtask 1d and PyMT tumor cells isolated from MMTV-PyMT mice using** nu/nu **hosts to monitor tumor development and metastasis (months 12-24).***

Data from Year 1 suggest that mammary fibroblasts isolated from *Atg12fl/fl;lsl-RFP+;FSP-Cre+* mice do not expand in culture, presumably because they are autophagy deleted and have a significant growth defect (data shown previously). The sufficient cell numbers impairs us from performing co-mixing experiments in mice. We decided to circumvent this issue by taking advantage of the *Atg12fl/fl;lsl-RFP+;PyMT+* mice (that do not carry *FSP-Cre*) for CAF isolation. To isolate these cells, mammary tumors from *Atg12fl/fl;lsl-RFP+;PyMT+*

**Figure 2: Evidence for efficient recombination within EpCAM negative cell populations of FSP-Cre+ mammary glands.**

**A.,** FACS analysis of *Atg12fl/fl;lsl-RFP+;FSP-Cre-* mammary glands, gated for RFP+ cells (R5). Note the lack of RFP+ cells in the R5 gate. **B.,** FACS analysis of *Atg12fl/fl;lsl-RFP+;FSP-Cre+* mammary glands, gated for RFP+ cells (R5). Note the large population of RFP+ cells in the R5 gate. **C.,** To confirm the RFP+ cell population in B is indeed stromal cells (FSP+) and not mammary epithelial cells, the R5 gate in B was further subgated into EpCAM- (stromal cells) or EpCAM+ cells (epithelial cells). Note the RFP+ fraction is EpCAM-.

mice were digested and sorted for PDGFRα+ and F4/80– cells to enrich for CAFs. The sorted CAF population was then immortalized with SV40 Large T Antigen to enable propagation of sufficient cell numbers for *in vivo* studies (Figure 3A, top). We further showed that immortalized CAFs sorted from *Atg12fl/fl;lsl-RFP+;PyMT+* mouse tumors were positive for fibroblast markers, negative for MEC markers, and able to be infected with Adenovirus expressing Cre recombinase, rendering them Atg12 deleted and autophagy deficient (data shown previously). We utilized this experimental design to co-mix autophagy competent or autophagy deficient CAFs with primary PyMT tumor cells and perform orthotypic injections in both C57B/6 host mice (syngeneic tumor model) and nu/nu host mice (immunodeficient tumor model). Autophagy deficient CAFs significantly suppressed the growth of PyMT tumor cells in C57B/6 mouse mammary glands compared to autophagy competent CAFs (Figure 3A, bottom left). Surprisingly, however, this phenotype was masked when the co-mix tumors were grown in nu/nu mouse mammary glands (Figure 3A, bottom right). These data suggest that the adaptive immune response is required for autophagy competent CAFs to mediate their tumor promoting effect.

Given that the tumor promoting phenotype was masked in immunodeficient mice, we wanted to investigate the extent of adaptive immune cell recruitment to the autophagy competent or deficient co-mix tumors in the C57B/6 mice. Using a CD3 antibody to delineate T cells, we noticed that the mammary tumors derived from PyMT cells co-mixed with autophagy deficient CAFs showed marked reduction in T cell

**Figure 3: Isolation and functional characterization of autophagy competent or deficient mouse cancer associated fibroblasts (CAFs). A.,** Top, schematic showing the isolation of CAFs from Atg12 homozygous floxed, PyMT+ C57B/6 mice. Bottom, average mammary tumor volume (mm$^3$) at endpoint of either autophagy competent (EV, red) CAFs co-mixed with PyMT cells, or autophagy deficient (Cre, blue) CAFs co-mixed with PyMT cells in C57B/6 (left) or nu/nu mice (right). Unpaired Student's t test (p=0.02 for C57B/6, not significant for nu/nu). **B.,** Histology of autophagy competent (EV) CAFs co-mixed with PyMT cells, or autophagy deficient (Cre) CAFs co-mixed with PyMT cells in C57B/6 mice. Top panel, H&E staining (20X); Bottom panel, CD3 immunohistochemistry (20X).

recruitment compared to control (Figure 3B). We are currently expanding this result to include more mice for statistical significance, as well as investigating the precise identity of the CD3 cells recruited (i.e., CD4+ and/or CD8+).

***Subtask 1f: Perform syngeneic transplants with autophagy competent (RFP+;FSP-Cre) and autophagy incompetent (Atg12fl/fl;RFP+;FSP-Cre and Atg5fl/fl;RFP+;FSP-Cre) mouse mammary fibroblasts (MMFs) into C57B/6 hosts, followed by inoculation with tumor cells 3 weeks following engraftment. (months 18-36)***

We modified the experimental design for this subtask for 2 important reasons: (1) As described in subtask 1e, MMFs isolated from *Atg12fl/fl;lsl-RFP+;FSP-Cre+* and *Atg5fl/fl;lsl-RFP+;FSP-Cre+* mammary glands do not grow out in culture, presumably because of a prominent growth defect due to autophagy deletion. Therefore, we cannot generate sufficient cell numbers to perform such studies. (2) Moreover, the *Atg12fl/fl;lsl-RFP+;FSP-Cre+* and *Atg5fl/fl;lsl-RFP+;FSP-Cre+* mice are still on a mixed genetic background, so we cannot do transplants into C57B/6 hosts.

To circumvent this issue, we utilized our Atg12 homozygous floxed, RFP+ mice on pure C57B/6 background to isolate MMFs. The mammary glands of *Atg12fl/fl;lsl-RFP+/-* or *lsl-RFP+/-* (control) mice were isolated and digested with collagenase and hyaluronidase enzymes. The cell suspension was then plated on type I collagen and grown in 10% calf serum to enrich for MMFs (protocol described in Year 1). After growth in culture for ~10 days, the MMFs were infected with Adenovirus expressing Cre recombinase and GFP (AdCre) to allow for recombination at the Atg12 and RFP loci (Figure 4A).
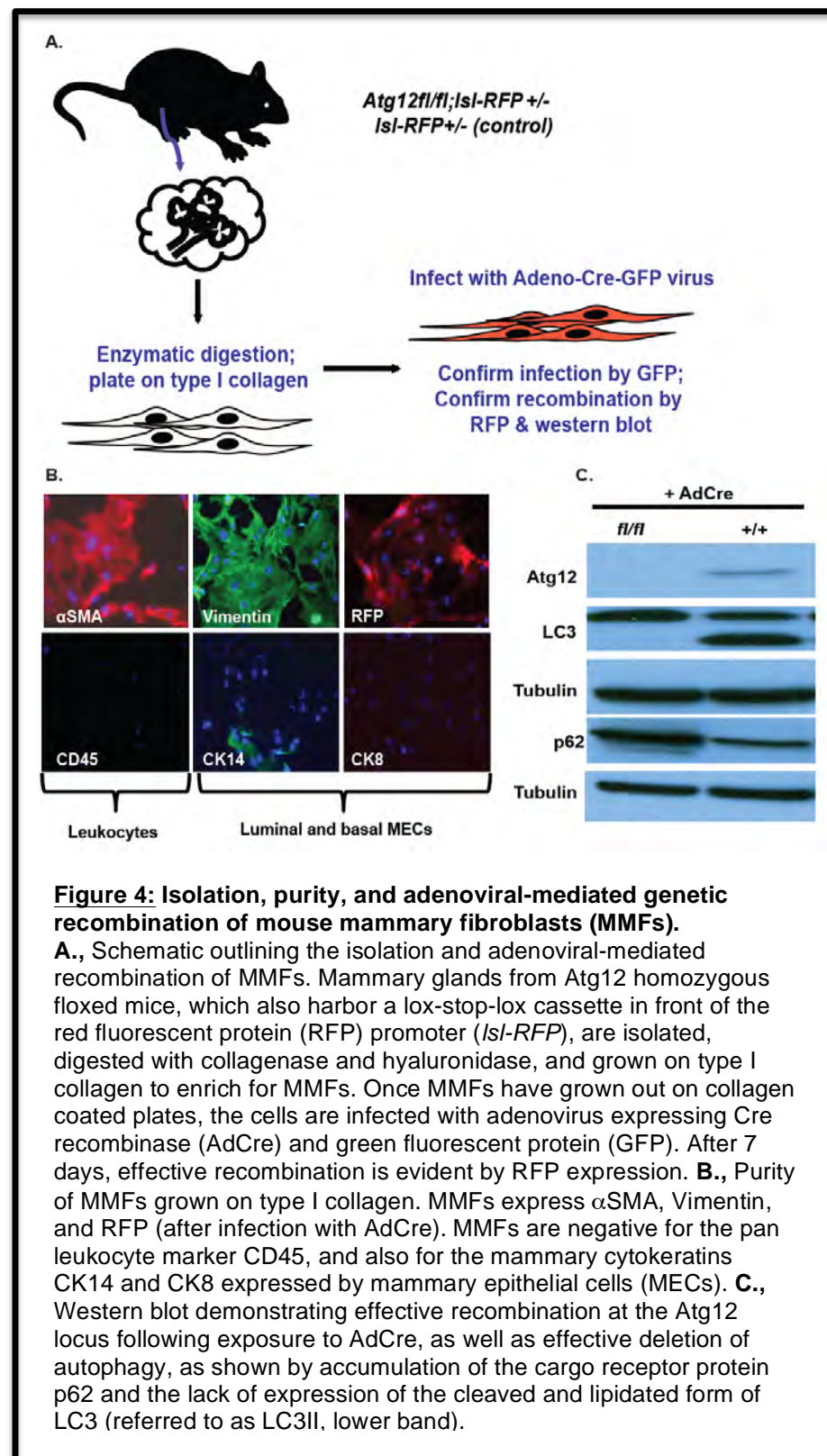
**Figure 4: Isolation, purity, and adenoviral-mediated genetic recombination of mouse mammary fibroblasts (MMFs).**
**A.,** Schematic outlining the isolation and adenoviral-mediated recombination of MMFs. Mammary glands from Atg12 homozygous floxed mice, which also harbor a lox-stop-lox cassette in front of the red fluorescent protein (RFP) promoter (*lsl-RFP*), are isolated, digested with collagenase and hyaluronidase, and grown on type I collagen to enrich for MMFs. Once MMFs have grown out on collagen coated plates, the cells are infected with adenovirus expressing Cre recombinase (AdCre) and green fluorescent protein (GFP). After 7 days, effective recombination is evident by RFP expression. **B.,** Purity of MMFs grown on type I collagen. MMFs express αSMA, Vimentin, and RFP (after infection with AdCre). MMFs are negative for the pan leukocyte marker CD45, and also for the mammary cytokeratins CK14 and CK8 expressed by mammary epithelial cells (MECs). **C.,** Western blot demonstrating effective recombination at the Atg12 locus following exposure to AdCre, as well as effective deletion of autophagy, as shown by accumulation of the cargo receptor protein p62 and the lack of expression of the cleaved and lipidated form of LC3 (referred to as LC3II, lower band).

Similar to our CAF isolations, we confirmed the purity of our MMF isolation after growth on type I collagen. MMFs were positive for fibroblast markers αSMA and Vimentin, positive for RFP after exposure to Cre recombinase, negative for the pan-leukocyte marker CD45, and negative for the mammary cytokeratins CK14 and CK8 (Figure 4B).
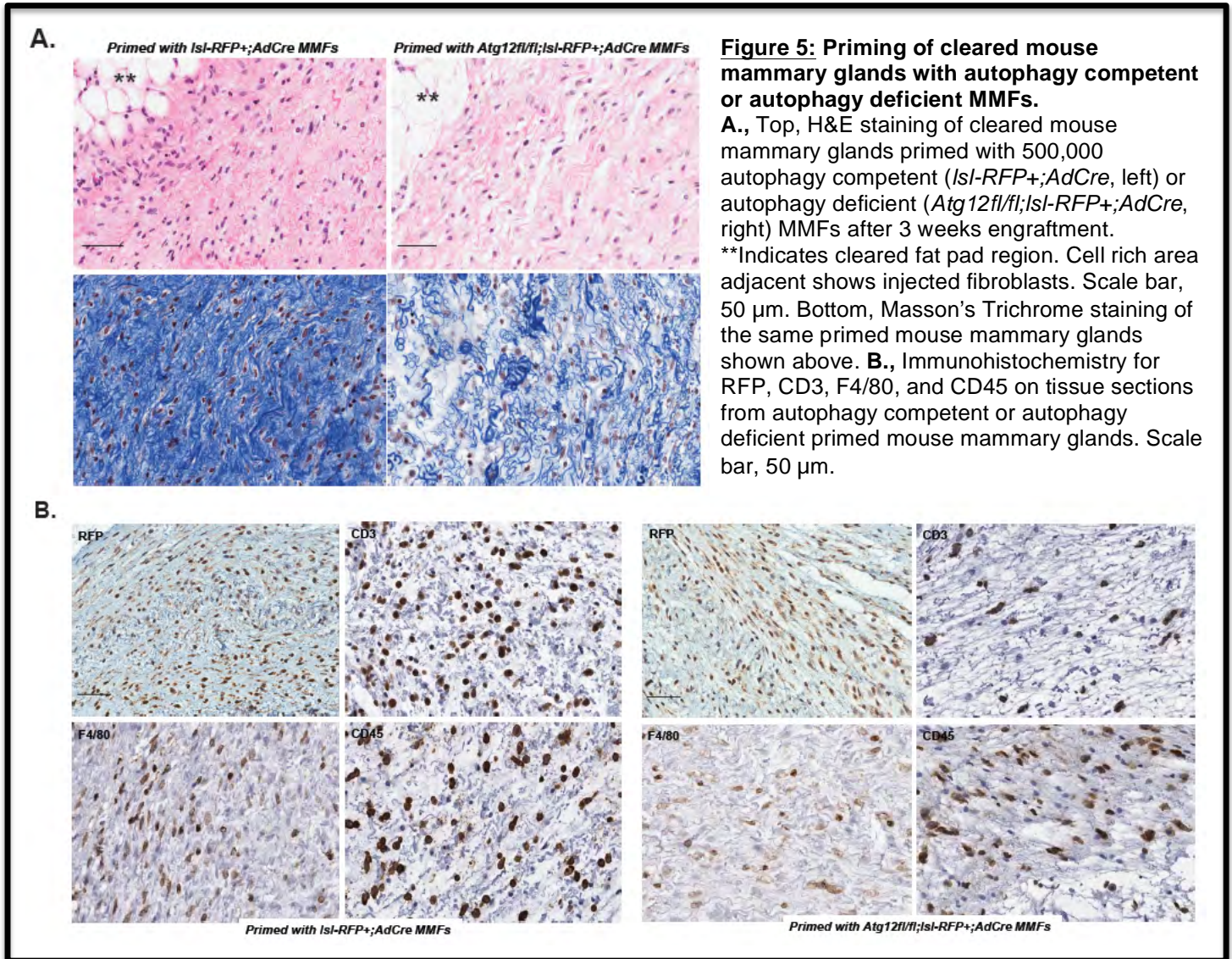
We confirmed that infection with AdCre rendered the *Atg12fl/fl;lsl-RFP+/-* MMFs autophagy deficient by generating protein lysate from these cells and western blotting for Atg12, LC3, and p62. As expected, *Atg12fl/fl;lsl-RFP+/-* MMFs infected with AdCre do not express Atg12 compared to their control cell counterparts (*lsl-RFP+/-* MMFs infected with AdCre). To confirm that *Atg12fl/fl;lsl-RFP+/-* MMFs infected with AdCre cannot undergo autophagy, we western blotted for expression of LC3-II (lower band), which is detected only in autophagy competent cells, and for expression of p62, which accumulates in autophagy deficient cells. As expected *Atg12fl/fl;lsl-RFP+/-* MMFs infected with AdCre do not express LC3-II and accumulate p62 as compared to their control cell counterparts (*lsl-RFP+/-* MMFs infected with AdCre; Figure 4C).

Using this MMF isolation and infection protocol, we were successfully able to engraft 500,000 autophagy competent or autophagy deficient MMFs into the "cleared" mammary glands of C57B/6 mice. Clearing the mammary gland (removing the mammary epithelium) allows the fibroblasts to engraft in the mammary fat pad, which is their native microenvironment. Thus, the gland consists of adipose tissue, the MMFs injected, and any other recruited immune cells; we refer to such a gland as "primed". H&E staining of the primed mammary fat pads after 3 weeks engraftment showed the bolus of MMFs injected, and Masson's Trichrome staining showed the collagen deposition (Figure 5A). Moreover, because the infected MMFs are RFP+, we confirmed that both the autophagy competent and autophagy deficient infected MMFs are still present in the fat pad after 3 weeks using an antibody against RFP (Figure 5B).

To address whether there was a difference in immune cell recruitment between the autophagy competent and autophagy deficient primed glands, we used antibodies against CD3 (a T cell marker), F4/80 (a macrophage marker) and CD45 (a pan-leukocyte marker). Our preliminary data indicate the total number of CD45+ cells recruited to the primed gland remains the same regardless of autophagy status, there are less CD3+ cells recruited in the autophagy deficient primed glands (Figure 5B). Moreover, our preliminary data indicates the total number of macrophages recruited to the primed gland also remains the same regardless of autophagy status. Despite similar total numbers of macrophages present in the primed glands, we are currently investigating if the polarization of the macrophage population skews differently depending on the autophagy status of the engrafted MMFs.
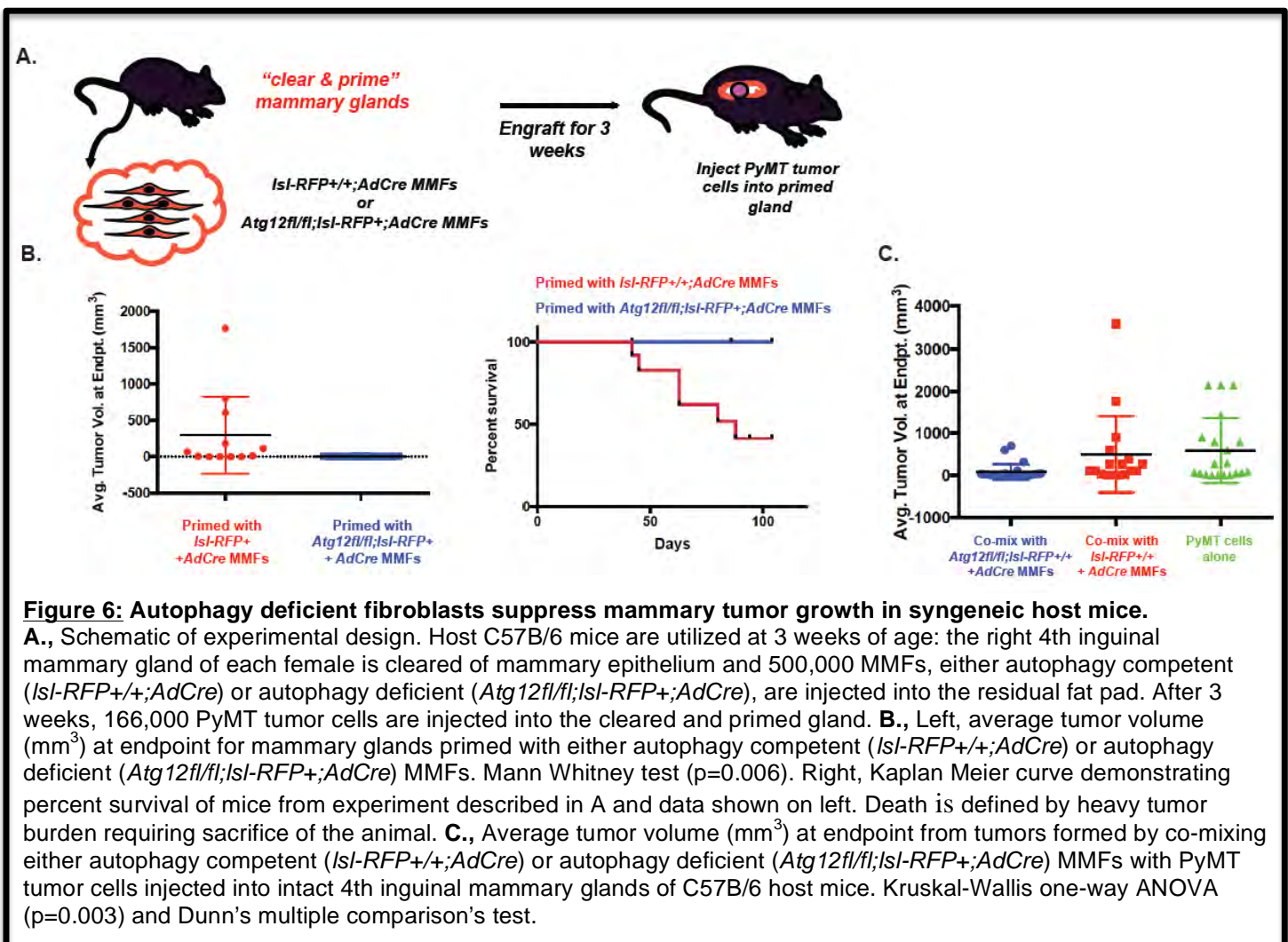
To address whether the autophagy competent or deficient primed mammary glands demonstrate differences in PyMT tumor growth, we injected 166,000 PyMT tumor cells into the primed region after 3 weeks



**Figure 5: Priming of cleared mouse mammary glands with autophagy competent or autophagy deficient MMFs.**
**A.,** Top, H&E staining of cleared mouse mammary glands primed with 500,000 autophagy competent (*lsl-RFP+;AdCre*, left) or autophagy deficient (*Atg12fl/fl;lsl-RFP+;AdCre*, right) MMFs after 3 weeks engraftment. **Indicates cleared fat pad region. Cell rich area adjacent shows injected fibroblasts. Scale bar, 50 µm. Bottom, Masson's Trichrome staining of the same primed mouse mammary glands shown above. **B.,** Immunohistochemistry for RFP, CD3, F4/80, and CD45 on tissue sections from autophagy competent or autophagy deficient primed mouse mammary glands. Scale bar, 50 µm.

engraftment (Figure 6A). Measuring tumor growth over ~12 weeks, we found that mice primed with autophagy deficient MMFs showed a significant impairment in PyMT tumor growth compared to mice primed with autophagy competent MMFs (Figure 6B). In fact, some of the mice primed with autophagy competent MMFs had prominent tumor burden requiring sacrifice of the animal at as early as 50 days, whereas mice primed with autophagy deficient MMFs failed to succumb to end stage tumor burden even at the termination of the study (Figure 6B).

To verify that the differences in tumor growth observed were not a consequence of the chosen experimental design, we used a well-established *in vivo* assay to demonstrate differences in fibroblast tumor promoting ability. Specifically, we co-mixed autophagy competent or autophagy deficient MMFs with primary PyMT tumor cells and injected the cells into intact 4th inguinal mammary glands of C57B/6 host mice. Again,

we observed that the autophagy deficient MMFs suppressed the growth of the PyMT tumor cells even in intact mammary glands (Figure 6C). These data suggest that the tumor suppressive ability of autophagy deficient MMFs is not contingent upon a cleared mammary gland or a 3 week engraftment period prior to tumor cell exposure. We are currently investigating the mechanism by which the autophagy suppresses the tumor promoting ability of MMFs.



**Figure 6: Autophagy deficient fibroblasts suppress mammary tumor growth in syngeneic host mice. A.,** Schematic of experimental design. Host C57B/6 mice are utilized at 3 weeks of age: the right 4th inguinal mammary gland of each female is cleared of mammary epithelium and 500,000 MMFs, either autophagy competent (*lsl-RFP+/+;AdCre*) or autophagy deficient (*Atg12fl/fl;lsl-RFP+;AdCre*), are injected into the residual fat pad. After 3 weeks, 166,000 PyMT tumor cells are injected into the cleared and primed gland. **B.,** Left, average tumor volume (mm$^3$) at endpoint for mammary glands primed with either autophagy competent (*lsl-RFP+/+;AdCre*) or autophagy deficient (*Atg12fl/fl;lsl-RFP+;AdCre*) MMFs. Mann Whitney test (p=0.006). Right, Kaplan Meier curve demonstrating percent survival of mice from experiment described in A and data shown on left. Death is defined by heavy tumor burden requiring sacrifice of the animal. **C.,** Average tumor volume (mm$^3$) at endpoint from tumors formed by co-mixing either autophagy competent (*lsl-RFP+/+;AdCre*) or autophagy deficient (*Atg12fl/fl;lsl-RFP+;AdCre*) MMFs with PyMT tumor cells injected into intact 4th inguinal mammary glands of C57B/6 host mice. Kruskal-Wallis one-way ANOVA (p=0.003) and Dunn's multiple comparison's test.

***Subtask 1g. Determine the latency period for the onset of primary tumor formation and metastasis for recipient mice generated in (f). At selected time points, 10 mice from each experimental cohort will be sacrificed and evaluated for characteristics of histopathologic progression from primary to metastatic disease (invasion, tumor cell proliferation/cell death, tumor burden, inflammatory cell recruitment, angiogenesis, and development/latency to metastasis). (months 24-36)***
These studies are planned for the upcoming year.
***Task 2: To determine if cytokines secreted by autophagy deficient FSP-Cre+ stromal fibroblasts influences mammary epithelial cell fate and behavior.***

***Subtask 2a. Use conditioned medium from cell populations isolated in Task (1d) to assess tumorgenicity of PyMT tumor cells*** in vitro *using a "tumorsphere assay." (months 12-30).*
These studies are planned for the upcoming year.

***Subtask 2b. Use conditioned medium from cell populations isolated in Task (1d) to assess invasive properties of PyMT tumor cells using a "scratch wound assay." (months 12-30).***
These studies are planned for the upcoming year.

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*Subtask 2c.* **Establish three-dimensional co-cultures using autophagy competent or incompetent CAFs isolated in Task (1d) with PyMT tumor cells and evaluate how autophagy inhibition in CAFs effects invasive behavior, basement membrane deposition, cell-cell junction integrity, mammary differentiation, proliferation and apoptosis. (months 12-36)**
      These studies are planned for the upcoming year.

*Subtask 2d.* **Obtain conditioned medium from cell populations isolated in Task (1d) to utilize for an unbiased cytokine array analysis, and validate potential candidates with mouse specific ELISAs. (months 24-30)**
      These studies are planned for the upcoming year.

*Subtask 2e.* **Cytokines of interest from the results in Task (2d) will be interrogated for their functional contribution to tumorsphere formation, proliferation, survival, and apoptosis. (months 24-36)**
      These studies are planned for the upcoming year.

*Subtask 2f.* **Determine the functional consequence of differential cytokine secretion between autophagy competent and incompetent mouse CAFs by generating shRNAs against potential cytokine candidates determined in Task (2d) and knocking down their expression in mouse CAFs. After selection, these CAFs will be functionally interrogated by co-mixing with PyMT tumor cells and subsequent injection into the MFPs of syngeneic recipient mice. I will use the same ratio of fibroblasts to PyMT cells as described in Task (1e). Tumor growth will be monitored over time as compared to mouse CAFs infected with a non-targeting shRNA control, and compared to injection of PyMT tumor cells alone. I anticipate 30 donor mice per cohort will be used for MMF and PyMT cell isolation, and 30 host recipient animals per cohort will be used for MFP injection. (months 18-36)**
      These studies are planned for the upcoming year.

## SUMMARY OF KEY RESEARCH ACCOMPLISHMENTS

1. To date, we have accumulated tumor growth data from sufficient numbers of mice with the following genotypes: *Atg12fl/fl;lsl-RFP+;FSP-Cre–;PyMT+* (autophagy competent); *Atg12fl/fl;lsl-RFP+;FSP-Cre+;PyMT+* (autophagy incompetent)*; Atg5fl/fl;lsl-RFP+;FSP-Cre–;PyMT+* (autophagy competent*); Atg5fl/fl;lsl-RFP+;FSP-Cre+;PyMT+* (autophagy incompetent). We have concluded that there are no significant differences in end stage tumor volume or tumor onset between *FSP-Cre+* and *FSP-Cre–* mice, despite ensuring Cre specificity in the mammary gland stromal (EpCAM–) cells.

2. We have successfully modified our experimental design to allow for (a) isolation and propagation of autophagy competent and autophagy deficient MMFs in culture; (b) engraftment of these cells into host C57B/6 mice for *in vivo* studies.

3. We have shown that autophagy deficient MMFs recruit less CD3+ cells in cleared/primed mouse mammary fat pads.

4. We have shown that autophagy deficient MMFs suppress fibroblast-mediated tumor promoting capability by: (a) clearing/priming C57B/6 host mammary fat pads with autophagy deficient or autophagy competent MMFs, followed by inoculation with PyMT tumor cells 3 weeks later; mammary glands primed with autophagy deficient MMFs show a significant reduction in end stage tumor volume and significant increase in survival when compared to their autophagy competent counterparts; (b) co-mixing autophagy deficient or autophagy competent MMFs with PyMT tumor cells and injecting these cells into intact mammary fat pads of C57B/6 host mice; autophagy deficient MMFs co-mixed with PyMT tumor cells show a significant reduction in end stage tumor volume compared to their autophagy competent counterparts; (c) co-mixing autophagy deficient or autophagy competent CAFs with PyMT tumor cells and injecting these cells into intact mammary fat pads of C57B/6 host mice; autophagy deficient CAFs co-mixed with PyMT tumor cells show a significant reduction in end stage tumor volume compared to their autophagy competent counterparts.

5. We have shown that autophagy deficient CAFs require an intact adaptive immune response to elicit their tumor suppressive ability, as this phenotype is masked in *nu/nu* mice.

## TRAINING AND PROFESSIONAL DEVELOPMENT

1. *U.S. Food & Drug Administration, Drug Development, Public Health & Science Policy course*
University of California, Berkeley
PB HLTH 236
January – April, 2015

2. *Scientific Leadership & Management for Postdoctoral Scholars*
University of California, San Francisco
2 day training, December 2014

## DISSEMINATED RESULTS

Nothing to report.

## IMPACT

Breast cancer is a disease of aberrant breast development whose etiology relies upon microenvironmental changes within the tissue. Such changes involve the appearance of $\alpha$-smooth muscle actin positive ($\alpha$SMA+) fibroblasts (CAFs), recruitment of various immune cells (macrophages, T cells, B cells, T regulatory cells), and enhanced type I collagen deposition. Within the last decade, a plethora of evidence demonstrates the importance of this inflammatory and desmoplastic stromal response to the initiation and progression of breast cancer. Despite this, the majority of cancer therapeutics fail to address this stromal response, largely exploiting the molecular mechanisms and genomic instability of breast cancer cells. Stromal gene expression data can predict patient response to pre-operative chemotherapy and has been implicated in breast cancer recurrence and metastatic disease. CAFs in particular serve as attractive drug targets in breast cancer, given their genomic stability and abundance in tumor associated stroma. However, the molecular mechanisms and cellular pathways that govern CAF behavior must be characterized before they can serve as potential targets for breast cancer therapeutics.

Importantly, CAFs are exposed to the same hypoxic and nutrient deprived microenvironments as tumor cells, and they likely upregulate stress response pathways in order to survive under these conditions. A commonly upregulated stress response pathway is autophagy, an evolutionarily conserved cellular stress response pathway that serves to remove damaged proteins and organelles and prevent cellular toxicity. Several cancer-relevant stresses induce autophagy, including nutrient deprivation, hypoxia, mechanical stress and chemotherapy. It remains unknown whether CAFs upregulate autophagy in order to survive in the tumor microenvironment.

Remarkably, the autophagy inhibitor hydroxychloroquine (HCQ) is already in phase I and II clinical trials as an adjuvant therapy for the treatment of metastatic breast cancer. However, it remains largely unknown how autophagy inhibition alters the behavior of stromal cell types, and how this ultimately impacts the growth and survival of breast tumor cells. *The studies outlined in this fellowship will provide essential information on whether autophagy inhibitors used clinically to treat breast cancer harbor stromal side effects that either work in unison or antagonistically with regression of tumor growth*.

## CHANGES/PROBLEMS

1. To date, we have accumulated tumor growth data from sufficient numbers of mice with the following genotypes: *Atg12fl/fl;lsl-RFP+;FSP-Cre–;PyMT+* (autophagy competent); *Atg12fl/fl;lsl-RFP+;FSP-Cre+;PyMT+* (autophagy incompetent)*; Atg5fl/fl;lsl-RFP+;FSP-Cre–;PyMT+* (autophagy competent)*; Atg5fl/fl;lsl-RFP+;FSP-Cre+;PyMT+* (autophagy incompetent). **We have concluded that there are no significant differences in end stage tumor volume or tumor onset between *FSP-Cre+* and *FSP-Cre–* mice**, despite ensuring Cre specificity in the mammary gland stromal (EpCAM–) cells. Because of this result, and the fact that these mice are still on a mixed genetic background, we cannot perform transplant studies with these mice as originally planned.

2. We have circumvented the issue described in (1) by isolating MMFs from our *Atg12fl/fl;lsl-RFP+* and *lsl-RFP+* mammary glands and infecting these cells with adenovirus expressing Cre recombinase to generate autophagy competent and autophagy deficient cell populations.

## PRODUCTS

Nothing to report.

## PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

| Name | Fanya Rostker | Hanna Starobinets |
|---|---|---|
| **Project Role:** | Animal Technician | Graduate Student |
| **Nearest person month worked:** | 5 | 2 |
| **Contribution to Project:** | Ms. Rostker has performed work in the area of subtask 1b and 1c. | Mrs. Starobinets has performed work in the area of subtask 1f |
| **Funding Support:** | 60% from DOD Era of Hope Scholar (W81XWH-11-1-0130) 40% from NIH R01 (CA188404) (both awarded to Jayanta Debnath) | National Science Foundation Graduate Research Fellowship Award (awarded to Hanna Starobinets) |

**<u>Syngeneic MFP Clearing & Transplantation</u>**

1.  *AT LEAST 3 WEEKS BEFORE THE DAY OF SURGERY,* sign up for the following:
    a.  Procedure room in 534
    b.  Isofluorane cart in main hallway (if using isofluorane as anesthesia)

2.  *2 1/2 WEEKS BEFORE THE DAY OF SURGERY,* isolate MMFs using described protocol.

3.  *10 DAYS BEFORE THE DAY OF SURGERY,* order the "host/recipient" mice from Charles River, Jackson Labs, or Harlan labs.

4.  *7 DAYS BEFORE THE DAY OF SURGERY,* perform Adeno-Cre infection using described protocol.

5.  *THE DAY BEFORE SURGERY*, prepare the following:
    a.  **Diluted Avertin (anesthetic, if not using isoflurane):**
        i.   Make a 10 ml 100% stock soln according to package instructions
        ii.  Dilute this 100% stock soln 1:40 in sterile saline in hood (2.5% final concentration)
        iii. Prepare enough volume for 2 avertin injections per mouse, with approximately 200 ul/injection (precise volume is dependent on the weight of the mice).
        iv.  Keep the avertin in a conical tube covered in foil on way to mouse house
        v.   Make fresh or use within one month of reconstitution
        vi.  WARM TO 37 °C before use- it will come out of soln!
    b.  **Diluted \*Bupernex (pain medication):**
        i.   Kept in the locked safe in the microscope room
        ii.  Key in Candia's drawer
        iii. Dilute 0.3 mg/ml ampule stocks 1:10 for a 0.03 mg/ml working stock soln in sterile saline in hood
        iv.  Transfer the remainder of ampule stock to a brown epindorf and label
        v.   Record all actions, volume used, etc in Bupernex drug log
        vi.  Surgical log that lists how much Bupernex is given to each mouse MUST match the remaining volume recorded in the drug log
    c.  **Diluted Carprofin (NSAID):**
        i.   Dilute 50 mg/ml stock soln to 1 mg/ml working soln in sterile saline in hood
        ii.  Make fresh or use within one month of reconstitution
    d.  **Weigh all mice that will be used for surgery and record how much of the Avertin, Bupernex and Carprofin they should receive. Record in surgical log.**
    e.  **List mouse numbers and the corresponding treatment per mouse in both notebook and on cage cards**
    f.  **In the evening, thaw matrigel on ice in cold room O/N.**

6.  *THE DAY OF SURGERY*, prepare cells for injection:
    a.  Prepare a 3:1 ratio of media:matrigel on ice (for a 1 ml soln, 250 ul matrigel and 750 ul media)
    b.  Trypsinize and count
    c.  Resuspend at $10^6$ cells/ml in media
    d.  Calculate how many cells are needed per genotype, based on the number of MFPs. Add 3 extra, to account for error in the syringe
    e.  Pipet up the appropriate volume corresponding to the appropriate number of cells/genotype, and spin down again

  f. Resuspend the pellet in the 3:1 mixture of media/matrigel, using a ratio of the volume of cell suspension to be injected per total number of cells injected per fat pad:
   Ex-  $6x10^6$ WT cells for (9+3 extra) MFPs; 500,000 cells injected per MFP
     $6x10^6$ WT cells * 20 ul cell suspension to inject / 500, 000 cells to inject = 240 ul to resuspend the pellet.
  g. Transfer cells to an epindorf and leave on ice

**Cell Counts/Resuspension info:**

7. *Bring the following from the lab to the Procedure Room:*
  a. Cells on ice
  b. Isoflurane kit (if using Iso as anesthesia)
  c. Diluted Avertin in a warm waterbath or already in soln; NOT ON ICE
  d. Diluted Bupernex on ice
  e. Diluted Carprofin on ice
  f. Surgical log and this protocol
  g. TID # sheets
  h. Blank slides labeled with TID #
  i. Pen
  j. Labeled cage cards
  k. Extra ice bucket for resected glands on slides

8. *Retrieve the cabinet key and isoflurane kit from Room 535.*

9. *Retrieve a recovery cage and new cages for mice post surgery, with gel packs, wet food, nestlets and a house.*

10. *To set up the room:*
  a. **Immediately plug in the bead sterilizer (takes 20 min to warm up).**
  b. Wipe down the counter with dilute bleach
  c. Set up two Styrofoam blocks
  d. Rinse syringe in acetone, water, and 70% ethanol, each 2X
  e. Set up surgical tools, betadine swabs, alcohol swabs, shaver, cauderizer, stapler and remover
  f. Set up an area for drug preparation (pipet tips and aids, insulin syringes, weight of mice and volume calculations)
  g. **If using isoflurane, set up procedure area and check to make sure there is enough oxygen (more than 700 PSI to be safe) for the entire duration of the surgery**

11. *During surgery, do the following:*
  a. Anesthetize with Avertin (1 injection, test foot pedal response, give a booster if necessary) or isoflurane
  b. Give eye ointment.

    c.   Shave the sides of the mouse
    d.   Make a Y incision.
    e.   Clear MFP: cauderize the connection between 4<sup>th</sup> and 5<sup>th</sup> mammary glands and bloods vessels, remove epithelium from lymph node to tissue edge and place on a glass slide for carmine staining (see mammary gland whole mount protocol)
    f.   Close incision with wound clips
    g.   Give local ointment over sutures
    h.   Inject Bupernex and NSAID ip
    i.   Place mouse in cage on heating pad or in front of heat lamp and wait for it to wake up.
    j.   Once the mouse is somewhat awake, place in another cage (final cage, labeled with cage card).

*12. The 3 days post surgery:*
    a.   Mice need to be monitored every day for 3 days post day of surgery.
    b.   Make sure incision is closed, not infected, and mice appear active and responsive
    c.   Provide extra wet food if necessary

13. *3 WEEKS AFTER DAY OF SURGERY:* inject PyMT cells using standard MFP injection protocol into the primed gland

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